

# Myeloperoxidase Expression as a Potential Determinant of Parthenolide-Induced Apoptosis in Leukemia Bulk and Leukemia Stem Cells<sup>[S]</sup>

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## ABSTRACT

Given that parthenolide (PTL) is an effective antileukemic agent, identifying molecular markers that predict response to PTL is important. We evaluated the role of myeloperoxidase (MPO) in determining the sensitivity of leukemia cells to PTL-induced apoptosis. In this study, the level of PTL-induced generation of reactive oxygen species (ROS) and apoptosis was significantly higher in the MPO-high leukemia cell lines compared with the MPO-low leukemia cell lines. Pretreatment of MPO-high leukemia cells with a MPO-specific inhibitor, 4-aminobenzoic acid hydrazide, or a MPO-specific small interfering RNA (siRNA) abrogated the PTL-induced ROS generation and apoptosis, indicating that MPO plays a crucial role in PTL-induced apoptosis in leukemia cells. PTL-induced apoptosis was accompanied by down-regulation of nuclear factor- $\kappa$ B, Bcl-xL, Mcl-1, X-linked inhibitor of apoptosis protein, and survivin and

selectively observed in primary acute myeloid leukemia (AML) cells expressing higher levels of MPO ( $\geq 50\%$ ) while sparing both AML cells with lower MPO and normal CD34-positive (CD34+) normal bone marrow cells. The extent of PTL-induced apoptosis of the CD34+CD38– cell fraction was significantly greater in the MPO-high AML cases, compared with the MPO-low AML ( $P < 0.01$ ) and normal CD34+ marrow cells ( $P < 0.01$ ). Nonobese diabetic/severe combined immunodeficient human leukemia mouse model also revealed that PTL preferentially targets the MPO-high AML cells. Our data suggest that MPO plays a crucial role in determining the susceptibility of leukemia cells to PTL-induced apoptosis. PTL can be considered a promising leukemic stem cell-targeted therapy for AML expressing high levels of MPO.

## Introduction

Parthenolide (PTL) is a sesquiterpene lactone and the active component of the medicinal plant feverfew (*Tanacetum parthenium*) (Knight, 1995). PTL contains both an

$\alpha$ -methylene- $\gamma$ -lactone ring and an epoxide group, which interact with the nucleophilic sites of many biological molecules (Bork et al., 1997). In addition to its well known antimicrobial and anti-inflammatory properties (Brown et al., 1997), PTL has been reported to inhibit cell growth and induce apoptosis in a variety of tumor cells (Wiedhopf et al., 1973; Woynarowski and Konopa, 1981; Ross et al., 1999; Zhang et al., 2004b). It is noteworthy that PTL has also been shown to effectively eradicate acute myeloid leukemia (AML) stem cells and progenitor cells in vitro while sparing normal hematopoietic stem cells (Guzman et al., 2005). Thus, PTL could be an attractive agent for

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**ABBREVIATIONS:** PTL, parthenolide; ABAH, 4-aminobenzoic acid hydrazide; AML, acute myeloid leukemia; DCFH-DA, dichlorodihydrofluorescein diacetate; DiOC6, 3,3'-dihexyloxycarbocyanine iodide; DMSO, dimethyl sulfoxide; DPBS, Dulbecco's phosphate-buffered saline; EGCG, epigallocatechin-3-gallate; FBS, fetal bovine serum; MAPK, mitogen-activated protein kinase; GST, glutathione S-transferase; IKK,  $\kappa$ B kinase; JNK, c-Jun N-terminal kinase; LSC, leukemia stem cells; MMP, mitochondrial membrane potential; MPO, myeloperoxidase; NAC, N-acetylcysteine; NOD/SCID, nonobese diabetic/severe combined immunodeficient; NF- $\kappa$ B, nuclear transcription factor  $\kappa$ B; PARP, poly(ADP)ribose polymerase; PE, phycoerythrin; PI, propidium iodide; ROS, reactive oxygen species; ScAc, succinylacetone; siRNA, small interfering RNA; XIAP, X-linked inhibitor of apoptosis protein; SP600125, anthra[1,9-cd]pyrazol-6(2H)-one; z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone; SB203580, 4-[4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-1H-imidazol-5-yl]pyridine; HRP, horseradish peroxidase; FITC, fluorescein isothiocyanate.

treating myeloid leukemia bulk and leukemia stem cells (LSC).

Several mechanisms have been shown to be involved in the antitumor effect of PTL, including the inhibition of nucleic acid synthesis (Woynarowski and Konopa, 1981), induction of mitochondrial dysfunction (Wen et al., 2002), disruption of intracellular calcium equilibrium (Zhang et al., 2004b), induction of G<sub>2</sub>-M phase arrest (Wen et al., 2002; Pozarowski et al., 2003), and sustained activation of c-Jun N-terminal kinase (JNK) (Nakshatri et al., 2004; Zhang et al., 2004a). In addition, PTL blocks signal transduction and the activators of transcription 3 (STAT3) phosphorylation (Sobota et al., 2000). PTL is a potent inhibitor of NF- $\kappa$ B activation through direct binding to I $\kappa$ B-kinase (IKK) (Hehner et al., 1999; Kwok et al., 2001; Zhang et al., 2004a). Most importantly, PTL induces cell death through the generation of intracellular reactive oxygen species (ROS) in a glutathione-sensitive manner (Wen et al., 2002; Zhang et al., 2004b; Guzman et al., 2005). Because malignant cells are highly susceptible to PTL-induced cell death (Wang et al., 2006), it is important to identify specific markers that indicate the susceptibility of leukemic cells to PTL-induced cell death to allow the effectiveness of PTL as an antileukemia agent to be predicted. Myeloperoxidase (MPO), a heme protein abundantly expressed in azurophilic granules (Eiserich et al., 2002), catalyzes the formation of hypochlorous acid, a potent oxidant implicated in killing bacteria (Bryk et al., 2002). The MPO tissue destruction occurs through induction of necrosis and apoptosis (Wagner et al., 2000; Klebanoff, 2005). Expression of the MPO molecule is specific for myeloid precursors and their leukemic counterparts. Evidence of the functional significance of the MPO molecule, in addition to its diagnostic importance, in myeloid leukemia cells is accumulating. The percentage of MPO-positive blast cells is highly associated with clinical outcomes of AML (Takubo et al., 1983; Matsuo et al., 2003). The Japan Adult Leukemia Study Group study demonstrated that AML patients with 50% or higher MPO-positive blast cells have a significantly better outcome (Matsuo et al., 2003), although the biological significance of the MPO molecule as a prognosis-determining factor in AML is unknown. Recently, it has been shown that MPO is a key regulator of oxidative stress-mediated apoptosis in myeloid leukemia cells (Nakazato et al., 2007). ROS is the direct mediator of green tea polyphenol (–)-epigallocatechin-3-gallate (EGCG)-induced apoptosis in MPO-positive leukemia cells, whereas EGCG fails to induce apoptosis in MPO-negative leukemia cells (Nakazato et al., 2007). Therefore, MPO may be an important determinant of myeloid leukemia cell sensitivity to oxidative stress-mediated apoptosis induced by several antileukemia agents.

The aim of this study is to evaluate whether MPO plays a critical role in PTL-induced apoptosis of leukemia cells. Therefore, the detailed molecular mechanism of PTL-induced apoptosis in relation to MPO expression was further investigated. It is noteworthy that the high susceptibility of leukemia cells to PTL-induced apoptosis is selectively documented in primary AML bulk cells, as well as CD34+CD38– AML LSC candidates expressing high levels of MPO. In contrast, PTL at a concentration that induces a substantial degree of apoptosis in the MPO-highly expressing leukemia cells does not affect survival of CD34-positive normal hematopoietic stem cells. According to our findings, MPO expression is a

critical determinant of PTL-induced apoptosis in leukemia cells. Thus, PTL can be considered as a promising LSC-targeted therapy for AML with a high level of MPO.

## Materials and Methods

**Cells, Reagents, and Culture.** U937, K562, HL60, Kasumi-1, NB4, KG1, OCI-AML3, and MOLM13 human leukemia cell lines (American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 medium supplemented with 25 mM L-glutamine (Cambrex BioScience, Walkersville, MD) and 10% (v/v) heat-inactivated fetal bovine serum (FBS; HyClone Laboratories, Logan, UT). Primary leukemic cells were isolated in bone marrow samples of untreated patients with AML. The isolation of CD34-positive cells from normal bone marrow was performed using the immunomagnetic microbead method (Kim et al., 2007). Specimens were collected under the Severance Hospital Institutional Review Board-approved clinical sample procurement protocols, and informed consent was obtained in accordance with the Declaration of Helsinki. Parthenolide [4xi-Germacra-1(10),11(13)-dien-12-oic acid, 4,5-epoxy-6.alpha.-hydroxy-,  $\gamma$ -lactone/C<sub>15</sub>H<sub>20</sub>O<sub>3</sub>, Calbiochem, San Diego, CA] was reconstituted in dimethyl sulfoxide (DMSO) to a stock concentration of 50 mM. Cells were resuspended at a density of  $2 \times 10^5$ /ml in a medium containing 10% FBS and incubated at 37°C in the presence of various concentrations of PTL for the indicated times, which were then harvested for subsequent analyses. Control cells were treated with equal amounts of the solvent. The caspase inhibitor z-VAD-fmk [benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone], an MPO inhibitor 4-aminobenzoic acid hydrazide (ABAH), and a p38 inhibitor, SB203580 (4-[4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-1H-imidazol-5-yl]pyridine), were purchased from Calbiochem. A heme biosynthesis inhibitor succinylacetone (ScAc) and JNK inhibitor SP600125 (anthra[1,9-cd]pyrazol-6(2H)-one) were obtained from Sigma-Aldrich (St. Louis, MO) and Biomol (Plymouth Meeting, PA), respectively. All experiments were performed in triplicate.

**Antibodies.** Rabbit anti-MPO polyclonal and mouse anti- $\alpha$ -tubulin monoclonal antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal antibodies against poly(ADP)ribose polymerase (PARP), XIAP, BID, tBID, Bak, PUMA, JNK, phospho(p)-JNK, p38, p-p38, survivin, as well as horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and HRP-conjugated goat anti-mouse IgG, were purchased from Cell Signaling Technology (Beverly, MA). Rabbit polyclonal antibodies against NF- $\kappa$ B (p65), Mcl-1, and Bcl-xL were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The mouse monoclonal antibodies against caspase-8, caspase-9, cytochrome c, and Smac/DIABLO, and rabbit polyclonal antibody against caspase-3 were obtained from PharMingen (San Diego, CA). To analyze human cell engraftment in the nonobese diabetic/severe combined immunodeficient (NOD/SCID) experiments, bone marrow cells were blocked with the anti-Fc receptor antibody 2.4G2 and labeled with antihuman CD45 and CD33 antibodies (BD Biosciences, San Jose, CA).

**Evaluation of MPO Expression.** MPO expression was evaluated using flow cytometry. For fixation and permeabilization of the cells, an Intraprep cell permeabilization kit (Beckman Coulter, Fullerton, CA) was used. The cells ( $5 \times 10^5$ ) were incubated for 30 min at room temperature with an anti-MPO antibody (Abcam, Cambridge, UK), and an isotype-matched mouse IgG served as the control. The cells were washed in cold DPBS at 4°C and resuspended in 5  $\mu$ l of FITC-labeled goat anti-mouse IgG antibody (PharMingen) for 15 min. To analyze MPO expression in CD34+CD38– AML cells, cells were labeled with anti-CD34-phycoerythrin (PE; BD Biosciences), anti-CD38-PerCP-Cy5.5 (PharMingen), and anti-MPO antibody for 30 min. The cells were washed in cold DPBS at 4°C (Dulbecco's phosphate-buffered saline) and resuspended in 5  $\mu$ l of FITC-labeled goat anti-mouse IgG antibody. Flow cytometric analy-

sis was conducted using a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, BDIS, San Jose, CA). The percentage of cells expressing MPO was defined on the gates set for the CD34+CD38- population.

**Measurement of MPO Activity.** Cells were harvested, washed with DPBS, and then homogenized in a phosphate buffer containing 1% hexadecyltrimethylammonium hydroxide and centrifuged at 4500g for 15 min at 4°C to assay for MPO in the supernatants. MPO activity was measured using an enzyme immunometric assay kit for human MPO (Assay Designs, Ann Arbor, MI).

**Stable Transfection of K562 Cells with Wild-Type or Mutant MPO cDNA.** For transfection of wild-type or mutant MPO cDNA to K562 cells, the nucleofection method was used (Kim et al., 2007). A transfection mixture was prepared by mixing 5 µg of DNA with 100 µl of human Nucleofector V solution (Amaxa Biosystems, Gaithersburg, MD). pCI-MPO wild-type and pCI-MPO/R569W mutant cDNAs were kindly provided by Dr. Yasushi Miyazaki (Nagasaki University, Nagasaki City, Japan), with the pCI empty vector used as a control. The cell suspension ( $2 \times 10^6$  cells/ml) was immediately electroporated using a Nucleofector instrument (program T-16; Amaxa Biosystems). After electroporation, cells were immediately cultured with a complete medium in six-well plates in a 37°C/5% CO<sub>2</sub> incubator. Stable transfectants (K562/control, K562/MPO, and K562/R569W) were selected as a single clone in the presence of 1 mg/ml G418 (Sigma-Aldrich).

**Transient Transfection of MPO siRNA.** MPO-specific small interfering RNA (siRNA) (5'-AGGCATCACCACCGTGTCTAA-3') was purchased from QIAGEN, Inc. (Valencia, CA). The cell suspension of  $2 \times 10^6$  leukemia cells was immediately transfected with MPO-specific siRNA using the Nucleofector instrument (Amaxa Biosystems) in accordance with the manufacturer's instructions. Immediately after electroporation, the cells were resuspended in a complete medium and incubated in a humidified 37°C/5% CO<sub>2</sub> incubator. Mock control siRNA transfection was also performed for control experiments. The cells were harvested after 24 h and used in subsequent experiments.

**Apoptosis Assay.** The annexin V assay was performed as described (Kim et al., 2007). Cultured cells were washed and incubated in 100 µl of binding buffer containing 5 µl of annexin V-FITC (PharMingen). The nuclei were counterstained with propidium iodide (PI), and the fraction of apoptotic cells was determined by flow cytometry. To identify apoptotic CD34+CD38- cells, cells were labeled with anti-CD34-PE-Texas Red (ECD; Beckman Coulter) and anti-CD38-PE (BD Biosciences) for 30 min. The labeled cells were subsequently resuspended in 100 µl of annexin V-binding buffer and incubated with annexin V-FITC and 7-aminoactinomycin (7-AAD; Beckman Coulter) for 20 min at room temperature before analysis by flow cytometry.

**Assessment of Loss of Mitochondrial Membrane Potential.** The loss of mitochondrial membrane potential (MMP) was monitored using DiOC<sub>6</sub> (Kim et al., 2007). For each condition,  $4 \times 10^5$  cells were incubated at 37°C for 15 min in 1 ml of 40 nM DiOC<sub>6</sub> (Calbiochem) and subsequently analyzed by flow cytometry. The control experiments documenting the loss of MMP were performed by exposing the cells to 5 µM carbamoyl cyanide *m*-chlorophenylhydrazone (Sigma-Aldrich), an uncoupling agent that abolishes MMP.

**Cell-Cycle Analysis.** Cells were fixed in 70% ethanol at -20°C for 16 h and resuspended in 1 ml of cell cycle buffer (0.38 mM/l sodium citrate, 0.5 mg/ml RNase A, 0.01 mg/ml PI) at a concentration of  $1 \times 10^6$  cells/ml. Cell-cycle analysis was carried out using a FACSCalibur flow cytometer equipped with ModFit LT 3.0 software (Verity Software House, Topsham, ME).

**Measurement of Intracellular Generation of ROS.** To assess the intracellular production of ROS, cells were treated with PTL alone or together with the antioxidant *N*-acetylcysteine [NAC; (*R*)-2-acetamido-3-sulfanypropanoic acid; Sigma-Aldrich]. After the treatment, the cells were incubated with 10 µM/l dichlorodi-

hydrofluorescein diacetate (DCFH-DA; Invitrogen, Carlsbad, CA), which is oxidized by cellular ROS and forms the fluorescent compound dihydrofluorescein. In addition,  $1 \times 10^5$  cells were stained with 10 µM/l DCFH-DA for 30 min at 37°C and then washed and resuspended in DPBS. The oxidative conversion of DCFH-DA to dihydrofluorescein was measured by flow cytometry.

**Measurement of Intracellular GSH Content.** Changes in total cellular glutathione (GSH) levels were determined using a glutathione assay kit (Sigma-Aldrich), in accordance with the manufacturer's instructions. PTL-treated cells were harvested and lysed with a  $1 \times$  lysis solution. Supernatant was added to each well of a 96-well plate, followed by the addition of 5 µl of glutathione *S*-transferase (GST), 2.5 µl of substrate solution, and assay buffer. After thorough mixing and incubation at 37°C for 60 min, fluorescence emissions at 478 nm were determined with a luminescence spectrometer (PerkinElmer Life and Analytical Sciences, Waltham, MA) after excitation at 390 nm.

**Western Blot Analysis.** After the cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 20 mM NaF, 1 mM EDTA, 5 mM EGTA, 15 mM sodium pyrophosphate, 1 mM benzamide, 0.1 mM phenylmethylsulfonyl fluoride, and 1% Nonidet P-40) and briefly sonicated, mitochondrial and cytosolic fractionation were performed using a cytosolic/mitochondria fractionation kit (Oncogene Research Products, San Diego, CA). Protein yields were quantified using the Bio-Rad detergent-compatible protein assay kit (Bio-Rad, Hercules, CA). Equivalent amounts of protein (20 µg) were boiled for 10 min and resolved on a 15% polyacrylamide gel by SDS-polyacrylamide gel electrophoresis. The proteins were subsequently transferred onto nitrocellulose membranes (GE Healthcare, Little Chalfont, Buckinghamshire, UK), which were washed and incubated with the primary antibody for 2 h at room temperature. After washing, the membranes were incubated with the relevant HRP-conjugated secondary antibody (1:3000 diluted in Tris-buffered saline/Tween 20 containing 5% bovine serum albumin) for 1 h. After the membrane was washed four times in Tris-buffered saline/Tween 20, the reactive proteins were visualized using an enhanced chemiluminescence detection system (GE Healthcare).

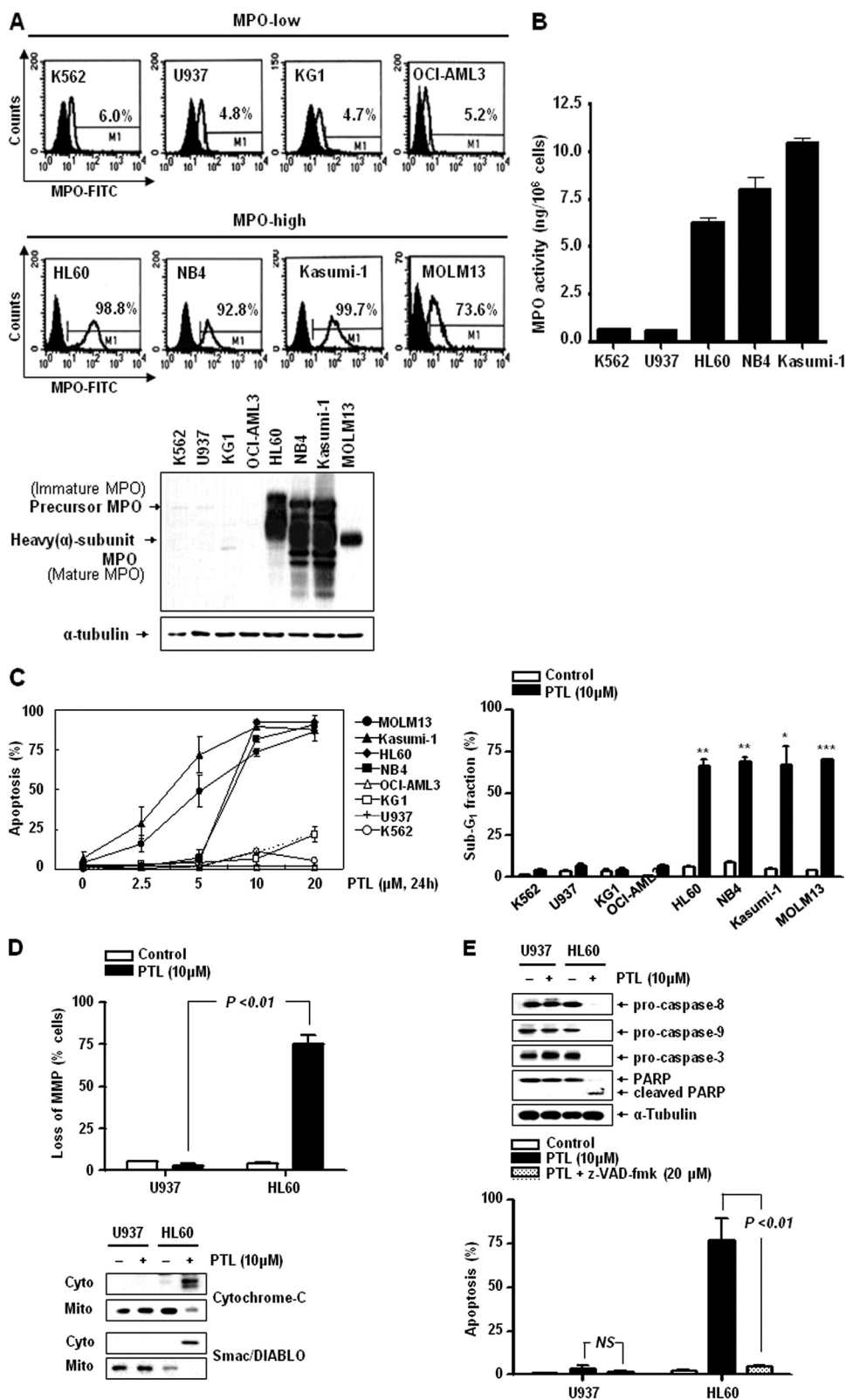
**NOD/SCID Human Leukemia Mice Model Assay.** NOD/SCID (NOD.CB17-prdkc scid/J) mice (Jackson Laboratories, Bar Harbor, ME) were irradiated with 2.7 Gy using a RadSource X-ray irradiator (RadSource, Boca Raton, FL) 1 day before transplantation. Human leukemia cells to be assayed were injected using the tail vein ( $5-10 \times 10^6$  cells) in a final volume of 0.2 ml of phosphate-buffered saline containing 0.5% FBS. After 6 to 8 weeks, the animals were sacrificed, and their bone marrow was analyzed for the presence of human cells by flow cytometry.

**Statistical Analysis.** Primary AML cases were divided into two groups based on the expression level of MPO protein in the leukemia cells: MPO-low and MPO-high. We arbitrarily defined primary AML cases as MPO-high and MPO-low AML according to whether the fraction of leukemic blasts expressing MPO was greater and lesser than 50% of the total mononuclear cells, respectively. The comparisons among the characteristics between groups were made using the  $\chi^2$  test for binary variables and the Mann-Whitney test for continuous variables. The statistics were calculated using SPSS software, version 11.0.1 (SPSS, Inc., Chicago, IL), with  $P < 0.05$  considered as significant.

## Results

**MPO Expression and Activity in Leukemia Cells.** MPO expression was examined in several leukemia cell lines using flow cytometric analysis. Whereas MPO expression was observed to be low in the K562, U937, KG1, and OCI-AML3 cell lines, MPO was highly expressed in the HL60, NB4, Kasumi, and MOLM13 cell lines (Fig. 1A, top). This was confirmed by Western blot analysis as the heavy ( $\alpha$ )-





**Fig. 1.** Differential sensitivity of leukemia cell lines to PTL-induced apoptosis according to MPO expression. **A**, constitutive expression of MPO protein in leukemia cell lines was examined by flow cytometry (top) and Western blot analysis (bottom) using the appropriate antibodies. **B**, MPO activity of the leukemia cell lines was measured using a human MPO enzyme immunometric assay as described under *Materials and Methods*. **C**, MPO-low cell lines (K562, U937, KG1, OCI-AML3) and MPO-high cell lines (HL60, NB4, Kasumi-1, MOLM13) were exposed to the indicated concentration of PTL for 24 h, and then the percentage of apoptotic cells was determined by annexin-V/PI staining using flow cytometry (left). Cells were cultured with 10 μM PTL for 24 h, fixed with 70% Et-OH, and stained with PI, after which the percentage of sub-G<sub>1</sub> populations was determined by flow cytometry (right). **D**, after treatment with 10 μM PTL for 24 h, the disruption of MMP was measured by DiOC<sub>6</sub> incubation and flow cytometry analysis in U937 and NB4 cells as described under *Materials and Methods* (top). Cytoplasmic translocation of cytochrome c and Smac/DIABLO protein was evaluated by Western blot analysis of the cytoplasmic and mitochondrial fraction (bottom). **E**, cleavage of caspases-8, -9, -3, and PARP was examined in U937 and HL60 cells 24 h after PTL treatment using Western blot analysis (bottom). The cells were exposed to 10 μM PTL for 24 h in the presence or absence of caspase inhibitor z-VAD-fmk (20 μM), after which the percentage of annexin V-positive apoptotic cells was determined by flow cytometry (bottom). Note that α-tubulin served as a loading control. Columns, mean value of three independent experiments; bars, S.D. Cyto, cytoplasmic fraction; Mito, mitochondrial fraction. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; NS, not significant.

subunit of mature MPO was observed in the HL60, NB4, Kasumi and MOLM13 cell lines but not in the K562, U937, KG1, and OCI-AML3 cell lines (Fig. 1A, bottom). In addition, as shown in Fig. 1B, MPO activity was remarkably higher in the HL60, NB4, and Kasumi cell lines compared with both of the K562 and U937 cell lines.

**PTL-Induced Apoptosis According to MPO Expression.** Apoptosis of leukemia cells induced by various concentrations of PTL was measured. The sensitivity of leukemia cell lines to PTL-induced apoptosis was highly dependent on the level of constitutive MPO expression. A limited degree of apoptosis was observed in U937 ( $9.7 \pm$

0.2%), K562 ( $11.0 \pm 0.1\%$ ), KG1 ( $6.2 \pm 1.1\%$ ), and OCI-AML3 ( $1.8 \pm 0.1\%$ ) after 24 h of treatment with  $10 \mu\text{M}$  PTL (Fig. 1C). However, the proportion of apoptotic cells in the HL60, NB4, Kasumi, and MOLM13 cell lines was markedly increased in a dose-dependent manner with PTL treatment. After 24 h of treatment with  $10 \mu\text{M}$  PTL, the mean proportion of apoptotic cells was  $92.5 \pm 0.7\%$  in HL60,  $81.5 \pm 1.1\%$  in NB4,  $89.2 \pm 0.8\%$  in Kasumi, and  $73.2 \pm 1.9\%$  in MOLM13 cell lines, which was remarkably higher than in the K562, U937, KG1, and OCI-AML3 cells (HL60 versus U937 or K562 or KG1 or OCI-AML3,  $P < 0.001$ ; NB4 versus U937 or K562 or KG1 or OCI-AML3,  $P < 0.001$ ; Kasumi versus U937 or K562 or KG1 or OCI-AML3,  $p < 0.001$ ; MOLM13 versus U937 or K562 or KG1 or OCI-AML3,  $P < 0.001$ ; Fig. 1C, left). These findings indicate that leukemia cells that express higher MPO levels are more sensitive to PTL-induced cell death. Significant increases in the proportion of leukemia cells in the sub- $G_1$  fraction after PTL treatment were limited to the high MPO-expressing cell lines (Fig. 1C, right).

The extent of MMP disruption induced by PTL was assessed by measuring the mitochondrial-mediated uptake of a membrane potential-sensitive dye DiOC<sub>6</sub>. The loss of MMP was significantly greater in HL60 cells compared with U937 cells (Fig. 1D, top), suggesting that mitochondrial-mediated apoptotic cell death induced by PTL occurred preferentially in leukemia cells that express higher level of MPO. PTL-induced cytoplasmic translocation of cytochrome *c* and Smac/DIABLO (Fig. 1D, bottom) and the cleavage of caspases-3, -8, -9, and PARP (Fig. 1E, top) were also limited to HL60 cells. In contrast, cytoplasmic translocation of cytochrome *c* and Smac/DIABLO and cleavage of caspase cascades in U937 cells did not occur after PTL treatment. Pretreatment of HL60 cells with  $20 \mu\text{M}$  of a pan-caspase inhibitor, z-VAD-fmk, led to the abrogation of PTL-induced cell death (Fig. 1E, bottom). These results indicate an association between expression of MPO and susceptibility to PTL-induced apoptosis, with this mechanism caspase-dependent.

**PTL-Induced ROS Generation in Relation to MPO Expression.** To explore whether MPO is critically involved in PTL-induced oxidative damage in leukemia cells, PTL-induced generation of intracellular ROS was measured in various leukemia cell lines. Five hours after treatment with  $10 \mu\text{M}$  PTL, flow cytometric analysis showed that the mean relative ROS levels in the MPO-high HL60, NB4, and Kasumi cells were remarkably higher than those in the MPO-low K562 and U937 cells (Fig. 2A, left). This finding suggests that MPO-high cells produce more ROS than MPO-low cells with PTL treatment.

When leukemia cells were preincubated with 1 mM of the free radical scavenger L-N-acetylcysteine (L-NAC) 1 h before PTL treatment ROS generation (Fig. 2A, right) and apoptosis (Fig. 2B) were almost completely abrogated in HL60, NB4, and Kasumi cells. This abrogation clearly suggests that an elevated ROS is crucial for PTL-induced apoptosis in MPO-high leukemia cells. We also measured the alteration of GSH levels upon PTL treatment; significant GSH depletion was observed only in HL60, NB4, and Kasumi cells (Fig. 2C). In addition, L-NAC pretreatment of the MPO-high cell lines completely abrogates the PTL-induced GSH depletion, which did not occur in the MPO-low cell lines (Fig. 2C).

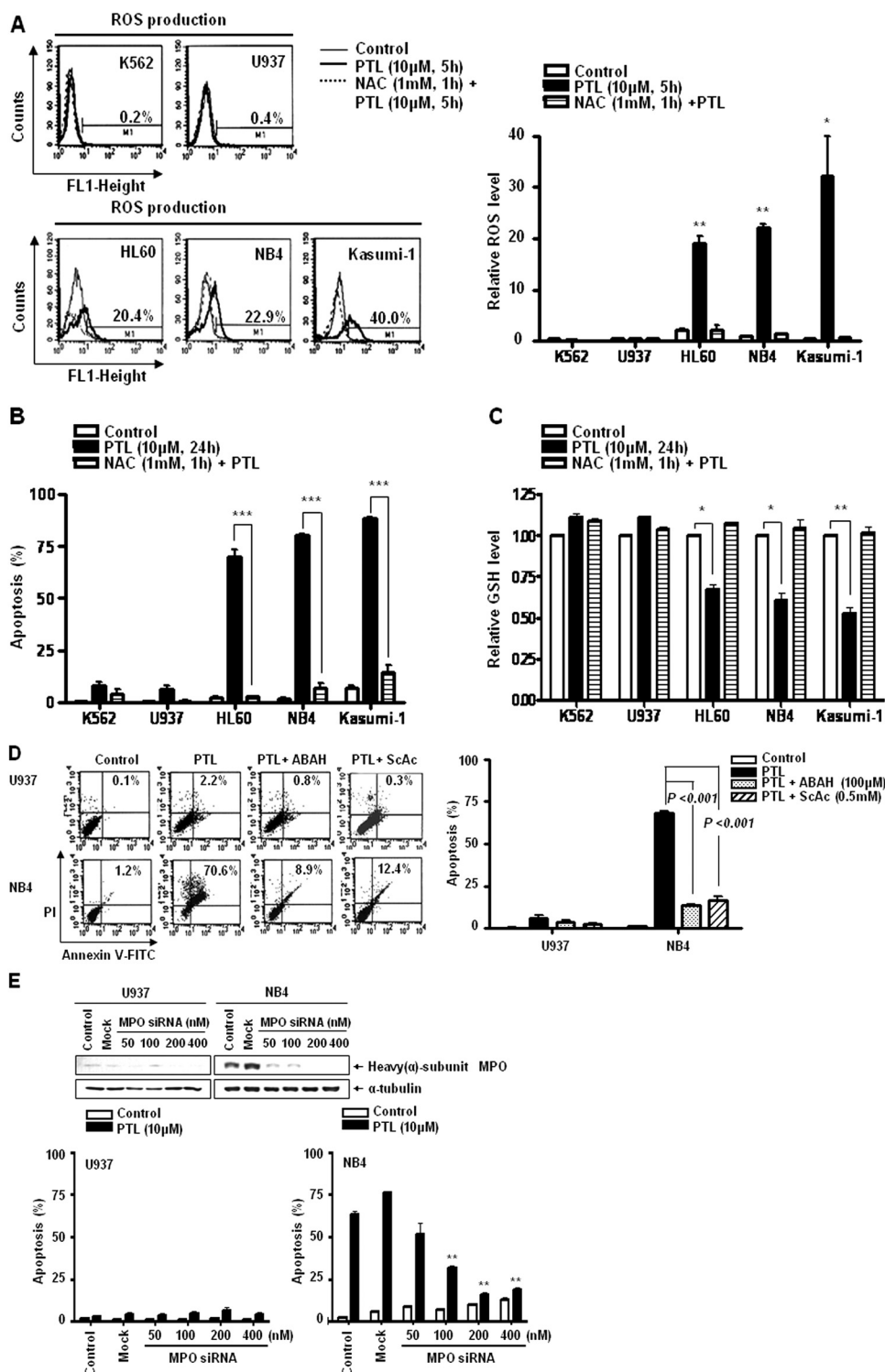
To elucidate the role of MPO in ROS-mediated apoptosis

that is induced by PTL, we evaluated the effect of the MPO-specific inhibitor ABAH and the heme biosynthesis inhibitor ScAc on PTL-induced apoptosis by flow cytometric analysis (Fig. 2D, left). Preincubation of NB4 cells with  $100 \mu\text{M}$  ABAH or 0.5 mM ScAc resulted in a significant inhibition of PTL-induced cell death (Fig. 2D, right). We further evaluated the effects of MPO-specific siRNA-mediated knockdown of MPO expression on the PTL-induced cell death. Western blot analysis demonstrated that MPO expression was effectively down-regulated in NB4 cells 24 h after transfection of cells with a MPO-specific siRNA, as described under *Materials and Methods* (Fig. 2E, top). MPO silencing significantly decreased the level of PTL-induced apoptosis in NB4 cells in a dose-dependent manner, whereas it was not observed in the control siRNA-transfected NB4 cells (Fig. 2E, bottom). Discernible effects were not seen in U937 cells. These findings clearly indicate that MPO activity plays a crucial role in PTL-induced cell death in leukemia cells.

**PTL-Induced Changes in the Apoptosis-Regulating Molecules According to MPO Expression.** Alterations in the levels of various antiapoptotic and proapoptotic molecules were evaluated after PTL treatment in leukemia cells. MPO-high HL60 cells showed a marked decrease in the levels of NF- $\kappa$ B (p65), Bcl-xL, Mcl-1, XIAP, and survivin after  $10 \mu\text{M}$  PTL treatment for 24 h (Fig. 3A). In contrast, these antiapoptotic molecules were not affected by PTL in MPO-low U937 cells. BID cleavage, Bak induction, and the presence of PUMA were observed only in HL60 cells with PTL treatment (Fig. 3A). A greater extent of phosphorylation JNK (p-JNK) was also observed in HL60 cells (Fig. 3B, top). However, p38 MAPK and phospho-p38 MAPK were not altered in either U937 or HL60 cells. Preincubation of HL60 cells with  $10 \mu\text{M}$  JNK-specific inhibitor SP600125 for 3 h abrogated PTL-induced cell death (Fig. 3B, bottom), suggesting that PTL-induced cell death in HL60 cells occurs through the JNK pathway. However, pretreatment of HL60 or U937 cells with the p38-specific inhibitor SB203580 did not affect PTL-induced cell death (Fig. 3B, bottom).

**Induced Overexpression of Wild-Type MPO-Enhances PTL-Induced Apoptosis.** To determine whether MPO expression directly contributes to the sensitivity of leukemia cells to PTL-induced apoptosis, two stable transfected cell lines that overexpress wild-type MPO (K562/MPO) or mutant MPO (K562/R569W) were used. K562 cells transfected with a pCI empty vector were used as a control. As shown by the flow cytometric and Western blot analysis, the heavy ( $\alpha$ ) subunit MPO protein was highly expressed in K562/MPO cell line, although not in the parental K562, K562/control, or K562/R569W cell lines (Fig. 4A).

The percentage of apoptotic cells in K562/MPO cells ( $65.8 \pm 7.6\%$ ) 24 h after treatment with  $10 \mu\text{M}$  PTL was found to be significantly higher compared with parental K562 ( $5.7 \pm 1.2\%$ ,  $p < 0.05$ ), K562/control ( $6.1 \pm 1.3\%$ ,  $p < 0.05$ ), and K562/R569W cells ( $11.4 \pm 2.8\%$ ,  $p < 0.05$ ) (Fig. 4b1). The histograms show a greater extent of sub- $G_1$  populations in K562/MPO cells after PTL treatment (Fig. 4b2, left). The proportion of cells at the sub- $G_1$  phase was also significantly higher in K562/MPO ( $58.9 \pm 0.1\%$ ) compared with K562 cells ( $3.5 \pm 0.7\%$ ,  $p < 0.01$ ; Fig. 4b2, right). The loss of MMP observed 24 h after PTL treatment was also significantly higher in K562/MPO cells ( $45.0 \pm 8.7\%$ ) than in K562 cells ( $0.7 \pm 0.1\%$ ,  $p < 0.05$ , Fig. 4b3, left). PTL-induced cyto-



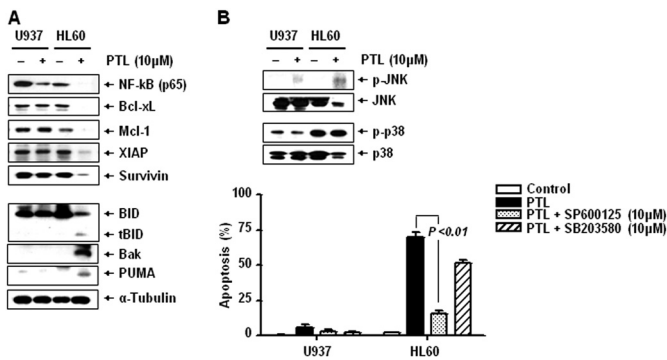
**Fig. 2.** PTL-induced ROS generation with depletion of GSH in relation to MPO expression. A, MPO-low cell lines (K562, U937) and MPO-high cell lines (HL60, NB4, Kasumi-1) were treated with 10  $\mu$ M PTL for 5 h in the presence or absence of prior treatment with 1 mM antioxidant NAC for 1 h. After incubation, intracellular ROS generation was measured using flow cytometric analysis as described under *Materials and Methods*. The representative histograms (left) and bar graphs (right) show a greater extent of ROS generation in MPO-high leukemia cell lines. Cells were treated with 10  $\mu$ M PTL for 24 h with or without NAC pretreatment, and then the fraction of apoptotic cells (B) and the intracellular GSH depletion (C) was evaluated using flow cytometry and luminescence spectrometer, respectively. D, U937 and NB4 cells were treated with PTL for 24 h in the absence or presence of ABAH (100  $\mu$ M, 24 h) or ScAc (0.5 mM, 3 h) pretreatment, and then the percentage of apoptotic cells was determined by flow cytometric analysis (left and right). E, U937 and NB4 cells were transfected with specific-MPO siRNA as described under *Materials and Methods*. Mock siRNA transfection was conducted as control experiment. After MPO gene silencing for 24 h, total cell lysates of U937 and NB4 cells were analyzed by Western blotting using antibody against MPO. MPO/ $\alpha$ -tubulin ratio according to amount of MPO siRNA was shown below the blots (top). The cells were treated with PTL for 24 h in the absence or presence of specific-MPO siRNA, and then the percentage of apoptotic cells was determined by flow cytometric analysis (bottom). Columns, mean of three independent experiments; Bars, S.D. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

plasmic translocation of cytochrome *c* and Smac/DAIBLO was observed only in K562/MPO cells (Fig. 4b3, right). Caspase-dependent apoptosis, which was abolished with 20  $\mu$ M z-VAD-fmk pretreatment before PTL addition, was confined to K562/MPO cells (Fig. 4b4). These findings suggest that MPO plays an important role in determining the sensitivity of myeloid leukemia cells to PTL-induced apoptosis through a mitochondria-mediated, caspase-dependent manner.

Pretreatment of K562/MPO cells with L-NAC pretreatment

completely inhibited the PTL-induced ROS generation, indicating that elevated ROS is crucial for PTL-induced apoptosis in K562/MPO cells (Fig. 4C, top). PTL-induced ROS generation was also significantly higher in the K562/MPO cells ( $31.5 \pm 1.0\%$ ) compared with parental K562 cells ( $2.4 \pm 0.3\%$ ,  $P < 0.01$ ) (Fig. 4C, bottom). Likewise, MPO overexpression contributed to PTL-induced GSH depletion. GSH depletion was significantly greater in the K562/MPO cells compared with K562 cells ( $P < 0.001$ , Fig. 4D). In contrast to the





**Fig. 3.** Alterations in the level of anti- or proapoptotic molecules with PTL treatment. A and B, top, MPO-low U937 and MPO-high HL60 cells were treated with 10  $\mu$ M PTL for 24 h, after which Western blot analysis was conducted using the appropriate antibodies. B, bottom, cells were exposed to PTL for 24 h with or without prior incubation with JNK inhibitor SP600125 or p38 inhibitor SB203580 for 3 h, and then the percentage of apoptotic cells was determined by flow cytometry. Columns, mean of three independent experiments; Bars, S.D.

parental K562 cells, treatment of K562/MPO cells with PTL for 24 h resulted in a marked decrease in the level of NF- $\kappa$ B, Bcl-xL, Mcl-1, XIAP, and survivin, indicating that MPO expression is directly associated with PTL-induced down-regulation of several antiapoptotic molecules (Fig. 4E). JNK phosphorylation was also more prominent in K562/MPO.

**Differential Sensitivity of Primary AML Cells to PTL According to MPO Expression.** We examined primary leukemic blasts obtained from patients with de novo AML and evaluated the correlation between the extent of PTL-induced cell death and their MPO expression levels (Table 1). Although MPO is an enzyme that is associated with myeloid lineage leukemia, variations in the proportion of MPO-positive leukemia cells were observed among the cases. As described in "Statistical Analysis," primary AML cases were arbitrarily defined as the MPO-high and MPO-low AML according to whether the fraction of leukemic blasts expressing MPO was greater or less than 50% of the total mononuclear cells. It was found that MPO activity was significantly higher in cases classified as MPO-high AML compared with MPO-low AML ( $P < 0.01$ ) and normal CD34+ bone marrow cells ( $P < 0.01$ ) (Fig. 5A). The average percentage of apoptotic cells induced by PTL treatment was also significantly higher in MPO-high AML cases ( $44.4 \pm 0.6\%$ ,  $n = 6$ ) compared with MPO-low AML cases ( $1.4 \pm 0.7\%$ ,  $n = 4$ ,  $P < 0.001$ ) and normal bone marrow CD34+ cells ( $8.8 \pm 0.5\%$ ,  $n = 4$ ,  $P < 0.001$ ) (Fig. 5B). The representative histograms show a greater extent of ROS production in MPO-high AML cells (Fig. 5C, top). PTL-induced increase in the relative ROS levels was also significantly higher in the MPO-high AML ( $24.4 \pm 1.6\%$ ) compared with MPO-low AML cases ( $5.9 \pm 0.3\%$ ,  $P < 0.001$ ) and normal bone marrow CD34+ cells ( $5.6 \pm 0.6\%$ ,  $P < 0.001$ , Fig. 5C, bottom). In addition, a significant level of GSH depletion was observed only in MPO-high AML (Fig. 5D). As a whole, these findings suggest that PTL is a potent antileukemic agent that potentially induces ROS generation and apoptosis in primary MPO-high AML cells while sparing normal hematopoietic stem cells and MPO-low AML cells.

**MPO Expression and PTL-Induced Apoptosis in CD34+CD38- LSC.** MPO expression in potential LSC candidate CD34+CD38- leukemic blasts from 14 un-

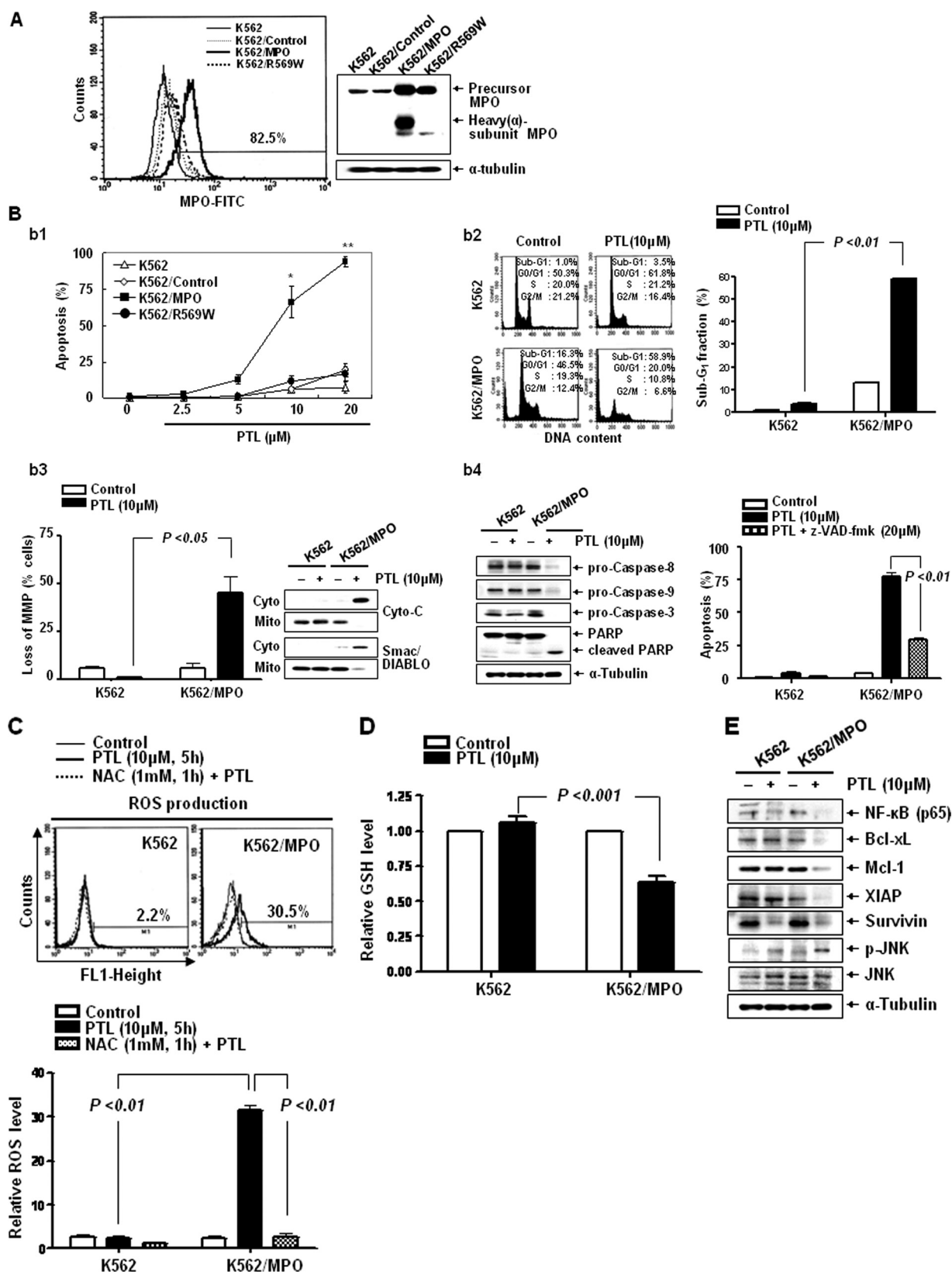
treated AML patients (supplemental data) were quantitatively measured based on the gated CD34+CD38- cell population by flow cytometry analysis. It is noteworthy that MPO expression was significantly higher in CD34+CD38- cells obtained from MPO-high AML cases than CD34+CD38- cells obtained from the MPO-low AML cases and normal bone marrow CD34+ cells (Fig. 6A, top, middle, and bottom left). The proportion of the MPO-positive CD34+CD38- cells in the MPO-high AML sample ( $80.2 \pm 5.5\%$ ) was significantly higher than that in the MPO-low AML sample ( $3.6 \pm 1.0\%$ ,  $P < 0.001$ ) and the normal bone marrow CD34+ cells ( $3.1 \pm 1.7\%$ ,  $P < 0.001$ ) (Fig. 6A, bottom right). The extent of apoptosis in CD34+CD38- leukemia cells induced by PTL treatment was also significantly greater in MPO-high AML cases ( $87.3 \pm 2.7\%$ ) compared with the MPO-low AML cases ( $32.5 \pm 6.4\%$ ,  $P < 0.01$ ) and the normal bone marrow CD34+ cells ( $26.0 \pm 11.5\%$ ,  $P < 0.01$ ) (Fig. 6B).

**NOD/SCID Human Leukemia Mice Model.** The NOD/SCID human leukemia mice model was used to further evaluate PTL-induced ablation of leukemia stem cells in relation to MPO expression. Primary leukemia cells were obtained from MPO-high AML and MPO-low AML cases, whereas CD34-positive bone marrow cells were obtained from healthy donors. These cells were preincubated with 10  $\mu$ M PTL for 24 h and transplanted into sublethally irradiated NOD/SCID mice. After 6 to 8 weeks, bone marrow cells were harvested and labeled with anti-human CD45 antibody to determine the percentage of human cells that were engrafted in each animal. As shown in Fig. 6C, PTL treatment significantly reduced the engraftment ability of primary leukemia cells that were harvested from the MPO-high AML cases compared with untreated MPO-high AML control cells. However, the engraftment potential of the PTL-treated primary leukemic blasts from the MPO-low AML cases was not decreased compared with the untreated control cells. The engraftment potential of normal CD34+ bone marrow cells did not change with PTL treatment in the NOD/SCID human leukemia mice model (Fig. 6C). As a whole, these results suggest that PTL is able to ablate the MPO-expressing AML bulk and LSC while sparing the normal functioning CD34+ hematopoietic progenitor cells.

## Discussion

PTL can be considered an attractive antileukemic agent, as it effectively eradicates AML cells and AML LSC in vitro while sparing normal hematopoietic stem cells (Guzman et al., 2005). In this study, we found the expression of MPO to be a crucial determinant of sensitivity to PTL-induced cell death in both myeloid leukemia cells and CD34+CD38- LSC candidates. The susceptibility of leukemia cells to PTL-induced apoptosis was significantly higher in the MPO-high leukemia cells and in the CD34+CD38- LSC obtained from MPO-high AML cases. The results also demonstrated that PTL does not affect the survival of the CD34+ normal bone marrow cells when used at a concentration that induces a substantial degree of apoptosis in the MPO-high leukemia cells. These findings suggest that PTL treatment may be a promising targeted therapy for MPO-high AML.

It was shown that PTL-mediated anticancer effects may be related to increased generation of ROS in cancer cells (Kwok



**Fig. 4.** Induced overexpression of MPO enhanced sensitivity to PTL-induced apoptosis. K562 cells were transfected with wild-type MPO (K562/MPO), mutant MPO (K562/R569W), and pCI empty control (K562/Control) cDNA construct using nucleofection as described under *Materials and Methods*. After electroporation, stable transfectant clones were selected and used for further experiments. **A**, heavy ( $\alpha$ ) subunit and precursor MPO protein expression in each transfectant cell lines were evaluated using flow cytometry (left) and Western blot analysis (right). **b1**, each transfectant was exposed to the indicated concentration of PTL for 24 h, and then the percentage of apoptotic cells was determined by flow cytometry. The histograms (**b2**, left) and bar graphs (**b2**, right) show the percentage of sub-G<sub>1</sub> populations measured by flow cytometry in K562 and K562/MPO cells 24 h after treatment with 10  $\mu$ M PTL. The disruption of mitochondrial membrane potential ( $\Delta\psi$ m) was measured by DiOC<sub>6</sub> incubation and flow cytometry analysis (**b3**,



TABLE 1

PTL-induced apoptosis of primary AML cells and normal CD34-positive bone marrow cells according to MPO expression

ID	Sex/Age	FAB Classification	MPO*	Apoptosis	
				Control***	PTL(10 $\mu$ M)**
			%		%
AML cases					
1	F/39	M1	18.4	0.3	3.5
2	M/39	M4	9.7	0.4	1.1
3	M/31	M2	4.1	0.8	0.1
4	M/69	M2	6.6	0.7	1.0
5	M/28	M2	48.2	0.6	44.2
6	F/33	M0	59.9	0.7	45.2
7	F/59	M1	66.0	0.1	45.6
8	M/65	M1	46.0	0.7	42.3
9	M/53	M4	46.7	0.8	43.0
10	F/25	M4	70.5	0.1	46.6
Healthy donors					
11	M/46		3.7	2.1	9.3
12	M/53		8.8	5.6	7.1
13	F/49		1.0	3.4	8.9
14	F/54		0.1	3.4	9.5

\* MPO(%), percentage of MPO-positive cells in the total mononuclear cells.

\*\* After treatment of primary AML cells or normal CD34-positive bone marrow cells with 10  $\mu$ M PTL for 24 h, the proportion of apoptotic cells was evaluated using flow cytometry.

\*\*\* Control, DMSO.

et al., 2001; Wen et al., 2002; Wang et al., 2006; Pajak et al., 2008). ROS induces apoptosis in a variety of cancer cells by stimulating proapoptotic signaling molecules, activating the p53 protein pathway, or initiating the mitochondrial apoptosis pathway (Wen et al., 2002; Zhang et al., 2004a,b) in a GSH-sensitive manner (Zhang et al., 2004b; Pajak et al., 2008). Therefore, the identification and attenuation of molecules that control the generation of ROS in leukemia cells could enhance their susceptibility to PTL-induced apoptosis.

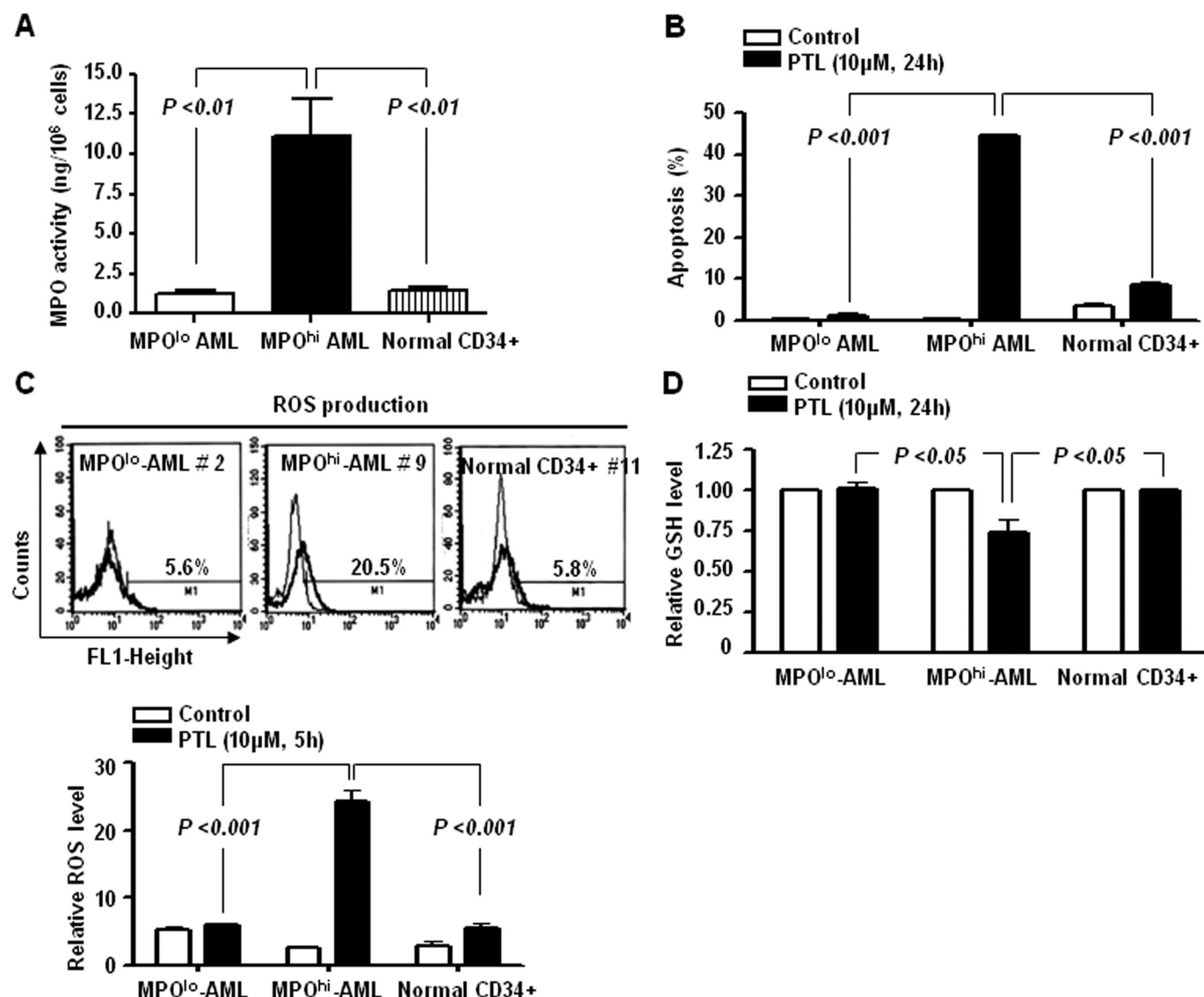
In this study, we demonstrated that PTL remarkably induces the generation of ROS and apoptosis specifically in both MPO-high leukemia cell lines and MPO-high primary leukemic cells obtained from AML patients. However, PTL failed to generate ROS or induce apoptosis in MPO-low leukemia cells. Pretreatment of leukemia cells with NAC abrogated PTL-induced ROS generation, GSH depletion, and cell death only in the MPO-high cells, suggesting that the generation of oxidant species is associated with PTL-induced cell death in the MPO-high leukemia cells. The MPO-specific inhibitor ABAH and the heme biosynthesis inhibitor ScAc also significantly inhibited PTL-induced apoptosis in MPO-high NB4 cells, whereas these MPO inhibitors did not affect PTL-induced apoptosis in MPO-low U937 cells. MPO silencing with MPO-specific siRNA transfection significantly decreased the level of PTL-induced apoptosis only in MPO-high NB4 cells in a dose-dependent manner. Furthermore, an induced overexpression of wild-type MPO gene in K562 cells led to a significant increase in the PTL-induced ROS generation, glutathione depletion, activation of caspase cascades, and mitochondrial pathway-mediated cell death compared with the overexpression in parental K562 cells. These findings clearly indicate that PTL-induced ROS generation and

apoptosis are critically associated with MPO expression in leukemia cells.

The specific ROS responsible for PTL-induced apoptosis in MPO-high leukemia cells was not investigated in this study. MPO is an endogenous lysosomal enzyme that removes  $H_2O_2$  and catalyzes the formation of toxic hypochlorous acid (HOCl) (Hampton et al., 1998; Klebanoff, 2005; Sawayama et al., 2008). HOCl interacts with other small molecules to produce various ROS, including hydroxyl radicals ( $\cdot OH$ ), singlet oxygen ( $^1O_2$ ), peroxynitrite ( $ONOO^-$ ), and ozone ( $O_3$ ) (Hampton et al., 1998). Previous studies have demonstrated that MPO-derived chlorinated oxidants and  $\cdot OH$  play key roles in inducing oxidative stress-mediated apoptosis in myeloid leukemia cells that are treated with EGCG (Nakazato et al., 2007), with ROS responsible for triggering apoptosis. It was suggested that  $H_2O_2$  produced in the presence of PTL is converted to HOCl by MPO. However, HOCl was not found to be responsible for directly inducing apoptosis as MPO-induced apoptosis was blocked by an  $O_2^-$  scavenger and an  $\cdot OH$  scavenger in MPO-positive leukemia cells (Nakazato et al., 2007). It can be suggested that a reaction between HOCl with  $O_2^-$  may result in the formation of a hydroxy radical that could directly induce apoptosis of MPO-positive leukemia cells.

NF- $\kappa$ B is a transcription factor that plays a key role in regulating cell proliferation, apoptosis, stress responses, and cell signaling pathways (Hayden and Ghosh, 2008; Baud and Karin, 2009). It was shown that PTL potentially inhibits NF- $\kappa$ B activity by inhibiting I $\kappa$ B kinase complex, resulting in sustained cytoplasmic retention of NF- $\kappa$ B (Bork et al., 1997; Suvannasankha et al., 2008). Because NF- $\kappa$ B is aberrantly activated in the CD34+CD38- LSC population and is associated with resistance to chemotherapy in AML (Guzman et

left), and the cytoplasmic translocation of cytochrome *c* and Smac/DIABLO protein examined by Western blot analysis (b3, right). The cleavage of caspases-8, -9, -3, and PARP was examined in two cell lines 24 h after PTL treatment using Western blot analysis (b4, left). K562 and K562/MPO cells were exposed to 10  $\mu$ M PTL for 24 h in the presence or absence of the caspase inhibitor z-VAD-fmk, after which the percentage of apoptotic cells was determined by flow cytometry (b4, right). K562 and K562/MPO cells were cultured with 10  $\mu$ M PTL for 5 h in the presence or absence of prior incubation with 1 mM NAC, after which intracellular ROS generation (C, top and bottom) and GSH depletion (D) were measured using flow cytometry. E, K562 and K562/MPO cells were treated with 10  $\mu$ M PTL for 24 h, and then Western blot analysis was conducted using the appropriate antibodies. Columns, mean of three independent experiments; Bars, S.D. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

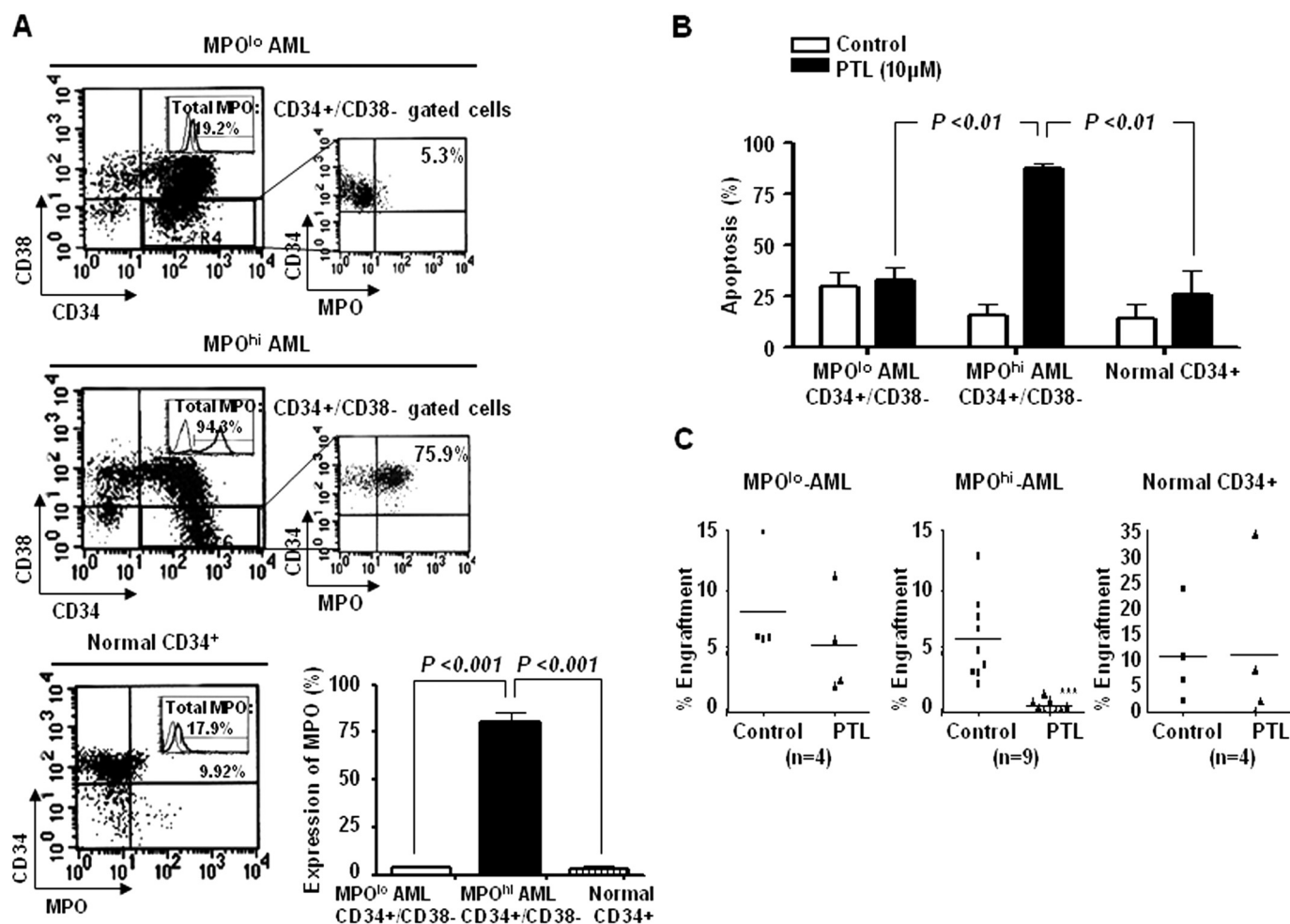


**Fig. 5.** Differential sensitivity of primary AML cells to PTL-induced apoptosis according to MPO expression. A, constitutive MPO activity of primary leukemia cells obtained from MPO-high(hi) AML ( $n = 6$ ), MPO-low(lo) AML ( $n = 4$ ) cases as well as CD34-positive normal bone marrow cells ( $n = 4$ ) was measured using human MPO enzyme immunometric assay. Primary MPO-high and MPO-low AML cases were arbitrarily defined according to the fraction of leukemic blasts expressing MPO as described under *Materials and Methods*. B–D, primary MPO-high AML cells, MPO-low AML cells, and CD34-positive normal bone marrow cells were treated with 10  $\mu$ M PTL for 24 h, and then the fraction of apoptotic cells (B) and intracellular ROS generation (C) were evaluated by flow cytometry (top and bottom), and GSH depletion (D) was examined using luminescence spectrometer. Columns, mean of three independent experiments; Bars, S.D.

al., 2001), it is possible that PTL could be used as an agent to preferentially eradicate LSC. However, we found that PTL inhibits NF- $\kappa$ B only in the MPO-high leukemia cells. Likewise, PTL-induced down-regulation of a variety of NF- $\kappa$ B-regulated antiapoptotic molecules, such as Bcl-xL, Mcl-1, the XIAP, and survivin (Khoshnan et al., 2000; Taguchi et al., 2006), were documented only in the MPO-high leukemia cells. PTL-induced truncation of the BH3-only protein Bid (tBid) and induction of the proapoptotic targets PUMA and Bak as well as phosphorylation of p-JNK were also observed selectively in the MPO-high leukemia cells.

LSC usually exists in a quiescent state and is therefore unlikely to respond to standard antileukemia chemotherapeutic agents that preferentially eradicate actively cycling cells (Holyoake et al., 1999; Graham et al., 2002; Guan et al., 2003; Guzman et al., 2005). The persistence of LSC after

chemotherapy may be a major factor contributing to relapse (Jordan and Guzman, 2004; van Rhenen et al., 2005). For these reasons, development of novel therapeutic agents that specifically target the LSC population without affecting normal hematopoietic stem cells is therefore crucial (Guzman et al., 2005). Because NF- $\kappa$ B is constitutively activated in the LSC population (Guzman et al., 2001), PTL can be considered a LSC-specific therapeutic agent. These primitive AML cells may be more sensitive to changes in oxidative stress than normal hematopoietic stem cells, and the resultant increase in ROS may contribute to AML-specific cell death (Guzman et al., 2005). As MPO was considered a crucial determinant of PTL-induced apoptosis in leukemia cells, we evaluated MPO expression in the primary AML cells with respect to CD34 and CD38 antigen coexpression. MPO was highly expressed in the CD34+CD38<sup>−</sup> leukemia cells obtained from the MPO-



**Fig. 6.** Differential sensitivity of CD34<sup>+</sup>CD38<sup>-</sup> leukemia stem cells to PTL-induced apoptosis according to MPO expression. **A**, representative flow cytometric dot plot analysis of CD34, CD38, and MPO expression in primary leukemia cells obtained from a MPO-low (top left), a MPO-high AML (middle left), and normal bone marrow CD34<sup>+</sup> case (bottom left). Histograms in the inner box showed the proportion of MPO-positive cells in the total leukemia cells in each case. MPO expression in the gated CD34<sup>+</sup>CD38<sup>-</sup> LSC fraction was determined in the MPO-low (top right) and a MPO-high AML case (middle right) using three-color flow cytometric analysis. Fraction of MPO-positive cells was significantly higher in the MPO-high AML ( $n = 10$ ) cases compared with MPO-low AML ( $n = 4$ ) cases and normal bone marrow CD34<sup>+</sup> cells ( $n = 3$ ) (bottom right). **B**, cells from MPO-high ( $n = 10$ ), MPO-low ( $n = 4$ ), as well as normal CD34<sup>+</sup> bone marrow cells ( $n = 3$ ), were treated with 10  $\mu$ M PTL for 24 h, and then the proportion of apoptotic cells in the CD34<sup>+</sup>CD38<sup>-</sup> LSC fraction and normal CD34<sup>+</sup> bone marrow cells was determined by flow cytometry as described under *Materials and Methods*. **C**, NOD/SCID mice were irradiated and transplanted with primary human leukemia cells obtained from MPO-high AML and MPO-low AML cases or with CD34<sup>+</sup> bone marrow cells harvested from healthy donors. Before transplantation, the cells were treated with 10  $\mu$ M PTL or DMSO control for 24 h. After 6 to 8 weeks, animals were sacrificed, and bone marrow was analyzed for the presence of human CD45<sup>+</sup> cells by flow cytometry. Each  $\blacksquare$  or  $\blacktriangle$  represents a single animal analyzed for level of human cell engraftment in the bone marrow. Mean engraftment is indicated by the horizontal bars.

high AML cases. In contrast, MPO expression was very low in the CD34<sup>+</sup>CD38<sup>-</sup> leukemia cells obtained from MPO-low AML cases. PTL-induced apoptosis of CD34<sup>+</sup>CD38<sup>-</sup> LSC candidates was significantly higher in MPO-high AML compared with MPO-low AML cases. This finding suggests that PTL has a selective effect on MPO-high CD34<sup>+</sup>CD38<sup>-</sup> LSC. PTL-induced apoptosis was negligible in the CD34<sup>+</sup> normal bone marrow cells in which MPO expression was very low.

Taken together, MPO molecule is demonstrated to be functionally related to PTL-induced apoptosis, ROS generation, GSH depletion, and the down-regulation of various antiapoptotic molecules, such as NF- $\kappa$ B in leukemia cells and CD34<sup>+</sup>CD38<sup>-</sup> LSC candidates. Therefore, the level of MPO expression within AML cells may be a potential marker for predicting the sensitivity of leukemia cells and LSC to PTL-induced cell death. Our findings indicate that PTL treatment can potentially be considered as a promising targeted ther-

apy for leukemia bulk and LSC in MPO-high AML cells. In addition, as conventional chemotherapeutic agents may induce NF- $\kappa$ B activation in leukemia cells, PTL can be combined with several common antileukemia drugs to overcome chemoresistance and enhance therapeutic responses.

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